Changes in Phenolic Composition of Apple Juices by HPLC with Direct Injection

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Abstract: An analytical procedure for the quantitative determination of the major polyphenols in apple juices which successfully employs a RP-HPLC system without prior treatment of the sample is described. Apple juices were clarified by microfiltration and ultrafiltration without previous treatment. Polyphenols monitored were influenced by clarification technology employed for stabilising the apple juices. The *p*-coumaric acid derivative was significantly affected by membrane type and filtration time, while (–)-epicatechin and phloridzin were influenced by filtration time. © 1998 Society of Chemical Industry.

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Key words: polyphenols; HPLC; direct injection; crossflow filtration; apple juices.

INTRODUCTION

Fresh apple juice is a delicate product from the chemical and microbiological point of view. Therefore, commercial juices must be preserved to produce a highly clarified juice, which does not show haze and/or sediments. Membrane techniques are simple to operate and automate and have low energy consumption and operating costs (Rao *et al* 1987; Constela and Lozano 1995); therefore, these techniques represent a viable alternative to the conventional process for the clarification of apple juices. Likewise, the problems associated with the elimination of fining agents and filter aids are minimum. These techniques have been optimised for the clarification of apple juices (Thomas *et al* 1987; Wu *et al* 1990; Giovanelli and Ravasini 1993; Padilla-Zakour and McLellan 1993).

Phenolic compounds are very important, due to their influence on the sensory quality of fruit juices (flavour, colour, bitterness and astringency) (Lea and Arnold 1978; Lea 1984; Amiot *et al* 1992). Polyphenols are also involved in changes of colour and development of undesirable haze and turbidity, which compromise

acceptability of commercial juices. These compounds are useful for determining adulterations of apple juices (Hermann 1990; Spanos and Wrolstad 1992).

Polyphenol analysis is generally accomplished by means of reversed-phase high-performance liquid chromatography (RP-HPLC) with UV-Vis detection. However, because of their complexity, a series of steps are usually taken to fractionate them (Jaworski and Lee 1987; Suárez Valles *et al* 1994; Suárez *et al* 1996), but these methods require tedious preliminary purifications and are time-consuming. A direct injection, with minimal pre-treatment of the sample, could be considered as an alternative when a great number of samples are to be analysed. This procedure has been assayed in wine (Roggero *et al* 1990, 1991; Lamuela-Raventós and Waterhouse 1994; Betés-Saura *et al* 1996) and fruit juices (Spanos and Wrolstad 1990; Spanos *et al* 1990). This option involves the selection of the appropriate variables to be measured and great care in the optimisation of the chromatographic separation of polyphenols.

In this paper, a method for analysing the major polyphenols in apple juice by direct injection is optimised and applied to determine the influence of different membrane clarification techniques on the phenolic composition of apple juices.

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TABLE 1

Gradient elution for the polyphenol separation in apple juices

<i>t</i> (min)	Flow rate (ml min ⁻¹)	%B
0	1.20	2
15	1.20	11
20	1.20	18.5
23	1.20	20.3
25	0.80	21.5
55	0.80	39.5

EXPERIMENTAL

Reagents

Standards of (–)-epicatechin, phloridzin, chlorogenic acid and *p*-coumaric acid were purchased from Sigma (St Louis, MO, USA). Procyanidins B1 and B2 and phloretin 2-xyloglucoside were kindly supplied by Dr A Lea (Reading, UK). Solvents employed were HPLC quality from Panreac (Barcelona, Spain) and degassed with helium prior to use.

Apple juices

Apple juice was prepared from a mixture of cider apple varieties, grown in the experimental orchard of the CIATA (Villaviciosa, Spain). The process includes milling with a grating mill and pressing with an hydraulic rack and frame press (30 min pressing time plus 15 h of maceration).

Pilot-scale, home-designed clarification equipment (Alvarez 1995) was used for obtaining the experimental apple juices. Clarification of the undepectinised apple juice was achieved using a back-pulsing, crossflow filtration technique and two size-exclusion membrane types (monochannel tubular modules of zirconium oxide with graphite support Carbosep®, from Techsep, Miribel, France), i.e. an ultrafiltration membrane with a nominal molecular weight cutoff of 50 kDa, and a microfiltration membrane of 0.14 µm mean pore size (hydraulic diameter 6 mm, filtration area 0.16 m²). Other conditions were as follows: pressure drop, 3.0–3.5 × 10⁵ N m⁻²; superficial velocity, 5.0–6.5 m s⁻¹; temperature, 20 and 50°C; sampling times 1 h, 2 h 30 min and 4 h. Experiments were carried out in triplicate. Samples were frozen at –20°C until analyses.

Preparation of the samples

Apple juices (containing 10 g litre⁻¹ of ascorbic acid) were filtered through a 0.45 µm cellulose acetate membrane filter (Teknokroma, Barcelona, Spain).

HPLC analysis

An HPLC system (Waters Chromatography Division, Milford, USA) equipped with a 712 automatic injector provided with a temperature controller, M510 pumps, a 996 diode array detector and Millennium v. 2.1 software data module was used. This software is provided with a system suitability testing option, which monitors chromatographic system performance. Resolution between peaks was automatically calculated by this program.

Samples were kept at 4°C and protected from light during the injection sequence. Separation of polyphenols was carried out on a reversed-phase Spherisorb hexyl cartridge (250 × 4.6 mm id, 5 µm particle size; Tracer Analítica, Teknokroma, Barcelona, Spain), at 34°C, using as solvents 2 mM phosphoric acid (solvent A) and methanol (solvent B). The gradient profile is described in Table 1.

Identification of polyphenols was achieved by comparing their spectra and retention times and by spiking with the available compounds under the same conditions.

Quantitation was done at 280 nm, by the external standard method. For those analytes where no standard was available, standards of the same family were used; thus, procyanidins were quantified as (–)-epicatechin, phloretin 2-xyloglucoside as phloridzin and the *p*-coumaric derivative as *p*-coumaric acid.

Statistical analysis

Analyses of variance were carried out by using the SPSS statistical package (SPSS 1988), considering an experimental design with three factors: filtration (two levels), temperature (two levels) and time (three levels). Repeated measures were carried out on the permeating time factor.

RESULTS

Condition optimisation for analysis

Three families of phenols were taken as references for the optimisation process: flavanols, hydroxycinnamic acids and dihydrochalcones, either because of their presence in high levels (like (–)-epicatechin, dihydrochalcones or chlorogenic acid) or their involvement in browning process (like chlorogenic acid or procyanidins B1 and B2). In apple juices obtained by pressing, flavanols are present in small quantities, as they are located only in the peel (Dick *et al* 1987; McRae *et al* 1990; Pérez-Illzarbe *et al* 1991); therefore, these compounds were not included in this study, although they could be successfully separated by direct injection in single-variety apple musts obtained with a domestic juicer-maker.

The chromatographic conditions have been optimised elsewhere for analysing phenolic neutral and acidic extracts (Suárez *et al* 1996); however, some modifications were necessary to improve the resolution between different compounds, eg (–)-epicatechin and the *p*-coumaric acid derivative, or hydroxycinnamic acids. From previous experiments (flow rates, different gradient shapes, temperatures), the authors have observed that only the temperature of analysis could be decisive in the analysis of polyphenols. Thus, this parameter was studied in the range from 30 to 35°C.

At 30°C, the resolution between (–)-epicatechin and the *p*-coumaric derivative was not sufficient (less than 0.8); increasing the temperature to 35°C, the resolution between the hydroxycinnamic acids (*p*-coumaric derivative and chlorogenic acid) (less than 0.6) became worse. In contrast, dihydrochalcones (phloretin 2-xyloglucoside and phloridzin) showed good resolution at any temperature, being slightly better at 35°C. Therefore, a compromise temperature of 34°C was selected for analysing major polyphenols in the apple juices, since separation was adequate in this case for all the compounds studied (Fig 1). The resolution values achieved by the optimised method are summarised in Table 2.

Accuracy and precision of analysis with direct injection

Precision of analysis was tested by injecting three times an apple juice sample. The relative standard deviations were comprised between 0.6 and 4.2%.

In order to test the reliability of the direct injection method, a recovery study was performed. Known amounts of (–)-epicatechin, chlorogenic acid and phloridzin were added in duplicate to an apple juice, and the resulting spiked samples were analysed. Recoveries ranged between 93 and 102% for all the compounds tested, as shown in Table 2.

Stability of the samples

Ascorbic acid (10 g litre⁻¹) was added to each sample to avoid polyphenol oxidation. However, differences

Fig 1. Effect of the temperature on the separation of polyphenols. Column Hexyl 25 cm × 0.46 cm, 5 µm. Mobile phase and gradient, see text. (A) 35°C; (B) 34°C; (C) 30°C. Peaks: 1, procyanidin B1; 2, (–)-epicatechin; 3, *p*-coumaric acid derivative; 4, chlorogenic acid; 5, procyanidin B2; 6, phloretin 2-xyloglucoside; 7, phloridzin.

were observed in the concentration of some phenols. Those differences could be related to the lapse of time passed once the samples were unfrozen. One sample was injected over 24 h, and the results for the phenolic compounds were collected and compared to the first analysis. The experiments performed showed that phenol concentrations were affected by time in different ways, as shown in Table 3. When analysis time exceeded a period of 13 h, the differences with regard to the first injection increased. Therefore, long injection sequences should be avoided.

Analysis in clarified apple juices

Major polyphenols quantified in clarified apple juices were as follows: procyanidins B1 and B2, (–)-epicatechin, *p*-coumaric acid derivative and chlorogenic acid, and the dihydrochalcones phloretin 2-xyloglucoside and phloridzin. Table 4 shows the mean

TABLE 2

Analytical characteristics of the direct injection method for phenolic compounds

Polyphenols	Resolution	% Recovery (± RSD)
(–)-Epicatechin	1.12	94.3 ± 1.8
<i>p</i> -Coumaric acid derivative	1.27	—
Chlorogenic acid		101.8 ± 0.9
Procyanidin B2		—
Phloretin 2-xyloglucoside	1.2	—
Phloridzin		93.1 ± 1.3

TABLE 3
Influence of time on the concentration of phenolic compounds in apple juices (mg litre⁻¹).

Time (h)	Procyanidin B1	(-) Epicatechin	p-Coumaric derivative	Chlorogenic acid	Procyanidin B2	Phloretin 2-xyloglucoside	Phloridzin
1	16.9	43.2	20.3	318.1	44.9	38.1	24.3
3	16.6	43.3	20.3	313.7	45.5	37.1	24.9
4	15.9	41.5	19.5	299.6	45.8	35.7	24.3
13	16.8	44.8	20.0	303.2	47.7	35.1	25.6
16	18.3	49.1	22.4	338.0	53.5	36.3	29.9
18	18.2	50.2	22.5	338.6	51.9	36.7	30.1

contents of phenolics for each filtration treatment and their correspondent standard deviations.

Chlorogenic acid was the main phenolic found in all the samples, in accordance with previous reports on dessert apples (Spanos *et al* 1990; Pérez-Illarbe *et al* 1991). Present results were even higher than those reported by Delage *et al* (1991) for industrial apple juices. This fact should be related to their analytical procedure, including liquid-liquid extraction with ethyl acetate, which yields poor extraction efficiencies for phenolic acids (Salagöity-Auguste *et al* 1984; Fernández de Simón *et al* 1990; Picinelli *et al* 1997).

The second major hydroxycinnamic acid exhibited a *p*-coumaric acid spectrum (maximum wavelength 312 nm), so that it was referred to as *p*-coumaric acid derivative and quantified as *p*-coumaric acid.

(-)-Epicatechin and procyanidin B2 had the highest levels among the neutral phenolic compounds.

Repeated measures analyses of variance applied to the phenolic contents in the stabilised apple juices showed that procyanidin B1, *p*-coumaric derivative, (-)-epicatechin and phloridzin were affected by some of the technological factors studied. Thus, significant differences were found for the procyanidin B1 content in

relation to the filtration treatment ($P < 0.05$) and the permeating time ($P < 0.01$). For this phenolic, interactive effects of technological factors (filtration and temperature) on the permeating time ($P < 0.01$) were ascertained. In Table 4, it can be seen that the evolution of procyanidin B1 during the clarification process depends on the filtration technique and temperature used; its content increased in all experiments, except for the juices stabilised by microfiltration at 20°C.

The *p*-coumaric derivative content was significantly affected by the technology of filtration and time ($P < 0.05$, $P < 0.1$). Worthy of note is the fact that interactive effects of the factors studied were not found. As seen in Table 4, this phenolic increased during the clarification process; moreover, the content of this compound was higher when microfiltration technique was employed.

(-)-Epicatechin and phloridzin contents were influenced by the filtration time ($P < 0.1$), with no interactive effects with other technological factors, which could be related to the concentration effect during permeating time.

In the present case, the filtration temperature does not have a pronounced effect, even though diffusion is

TABLE 4
Mean contents of phenolic compounds (mg litre⁻¹ ± SD) in clarified apple juices.

Filtration treatment ^a	Time (h)	Procyanidin B1	(-) Epicatechin	p-Coumaric derivative	Chlorogenic acid	Procyanidin B2	Phloretin 2-xyloglucoside	Phloridzin
MF (20°C)	1	14.1 ± 0.2	45.1 ± 4.3	11.8 ± 1.2	310.3 ± 28.4	36.4 ± 0.6	30.3 ± 3.1	17.5 ± 2.0
	2.5	12.8 ± 0.1	42.7 ± 3.6	14.6 ± 0.9	307.6 ± 33.0	33.2 ± 2.6	30.4 ± 3.1	17.3 ± 1.4
	4	12.5 ± 0.3	47.4 ± 2.7	14.7 ± 1.3	383.6 ± 25.2	39.7 ± 2.5	35.9 ± 3.5	19.7 ± 1.5
MF (50°C)	1	14.1 ± 0.2	38.1 ± 0.7	15.0 ± 0.9	271.6 ± 5.4	34.5 ± 0.8	27.4 ± 0.5	16.3 ± 0.8
	2.5	14.8 ± 0.4	39.6 ± 2.4	15.2 ± 1.3	290.1 ± 7.7	36.5 ± 1.1	28.7 ± 1.2	16.8 ± 1.2
	4	14.6 ± 0.2	40.8 ± 1.5	15.2 ± 0.1	308.0 ± 6.7	36.5 ± 0.2	30.2 ± 0.8	16.5 ± 0.1
UF (20°C)	1	15.6 ± 1.1	38.8 ± 1.8	11.4 ± 0.4	306.5 ± 8.1	37.2 ± 1.9	26.6 ± 1.0	12.3 ± 1.0
	2.5	16.3 ± 0.1	39.7 ± 0.8	12.3 ± 1.7	302.3 ± 6.6	37.4 ± 0.8	28.1 ± 1.2	15.5 ± 1.2
	4	17.3 ± 0.9	40.2 ± 0.8	14.0 ± 0.2	299.2 ± 8.2	38.1 ± 1.3	29.0 ± 2.1	17.6 ± 0.6
UF (50°C)	1	14.0 ± 0.9	34.7 ± 5.5	12.0 ± 2.0	292.2 ± 5.7	29.4 ± 1.5	30.1 ± 2.9	17.1 ± 1.2
	2.5	15.5 ± 0.9	41.2 ± 1.4	12.9 ± 1.3	313.0 ± 15.5	32.8 ± 3.0	30.3 ± 1.9	16.6 ± 0.5
	4	19.3 ± 1.2	45.3 ± 2.0	13.7 ± 1.4	335.1 ± 7.9	36.4 ± 2.6	32.5 ± 0.7	17.2 ± 0.4

^a MF, microfiltration; UF, ultrafiltration.

an important mechanism in membrane filtration process. However, Spanos *et al* (1990) observed an increase in apple juices obtained by diffusion at different temperatures. Membrane cut-off influenced the *p*-coumaric acid derivative, accounting for a higher retention of this compound by the ultrafiltration membranes, in agreement with the results of Giovanelli and Ravasini (1993).

CONCLUSIONS

The analysis of phenolic compounds in apple juices can be successfully accomplished by direct injection and RP-HPLC in terms of accuracy, rapidity and precision, in comparison with the results obtained by other techniques including isolation and fractionation steps.

Stabilisation of apple juices by membrane technologies significantly influenced the major phenolic profile, specially phloridzin, (–)-epicatechin, *p*-coumaric derivative and procyanidin B1. However, in the temperature range studied, effects of this factor on the phenolic level of apple juices were not found.

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